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Fas activates lipolysis in a Ca²⁺-CaMKII-dependent manner in 3T3-L1 adipocytes

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Running title: Fas-mediated lipolysis

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Abstract

Fas (CD95) is a member of the tumor necrosis factor (TNF) receptor superfamily and plays a crucial role in the induction of apoptosis. However, like TNF, Fas can induce non-apoptotic signaling pathways. We previously demonstrated that mice lacking Fas specifically in adipocytes are partly protected from diet-induced insulin resistance, potentially via decreased delivery of fatty acids to the liver as manifested by lower total liver ceramide content. In the present study we aimed to delineate the signaling pathway involved in Fas-mediated adipocyte lipid mobilization. Treatment of differentiated 3T3-L1 adipocytes with membrane-bound Fas ligand (FasL) significantly increased lipolysis after 12 hours without inducing apoptosis. In parallel, Fas activation increased phosphorylation of ERK1/2 and FasL-induced lipolysis was blunted in the presence of the ERK-inhibitor U0126 or in ERK1/2-depleted adipocytes. Furthermore, Fas activation increased phosphorylation of the Ca^{2+} /calmodulin-dependent protein kinases II (CaMKII) and blocking of the CaMKII-pathway (either by the Ca^{2+} chelator BAPTA or by the CaMKII inhibitor KN62) blunted FasL-induced ERK1/2 phosphorylation and glycerol release. In conclusion, we propose a novel role for CaMKII in promoting lipolysis in adipocytes.

Key words: obesity, insulin resistance, adipose tissue inflammation

Introduction

White adipose tissue (WAT) has major metabolic and endocrine functions mediated by secretion of different adipokines and fat-derived metabolites such as NEFAs. These molecules regulate food intake, energy expenditure, and glucose homeostasis (1, 2). In obesity, excess WAT accumulation is accompanied by local infiltration of macrophages and other inflammatory cells secreting different cytokines such as IL-1 α , IL-1 β , IL-6, IL-8 (KC) and MCP-1, which in turn alter the expression and secretion pattern of adipokines, cytokines, and stimulate the release of fatty acids by elevating basal lipolysis. All these changes contribute to the development of detrimental complications of obesity such as insulin resistance and diabetes mellitus (2, 3).

Lipolysis is physiologically stimulated by the catecholamine hormones noradrenaline and adrenaline through activation of protein kinase A (PKA). Further downstream, PKA activates hormone sensitive lipase (HSL), perilipin 1 and adipose triglyceride lipase (ATGL) resulting in triglyceride breakdown (4). Besides this well-established regulation of lipolysis by catecholamines, tumor necrosis factor alpha (TNF α) is another potent inducer of lipolysis in adipocytes (5). TNF α -mediated lipolysis involves activation of the p44/42 MAP kinases (ERK1/2) leading to down-regulation of the lipid droplet coating protein perilipin (6).

Fas (FasR, CD95, Apo-1) is a type I transmembrane protein that belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily (7) and is activated by Fas ligand (FasL, CD95L), a type II membrane protein (8). Fas was first described in 1989 as a surface molecule on lymphocytes that can trigger cell death (9). In the adult mouse, Fas and FasL are expressed in several tissues including WAT (10, 11). Upon binding of FasL, preformed trimeric Fas complexes undergo a conformational change that results in the formation of a death-inducing

signaling complex (DISC) and activation of downstream pathways leading to apoptosis (9, 12). However, in addition to this well-established role of Fas in apoptosis, Fas activation contributes to non-apoptotic signaling pathways, including cell proliferation (9, 13) and the induction of inflammatory responses in different cell types (14-18). Moreover, we have recently reported that Fas is increasingly expressed in WAT of obese subjects and that Fas-deficient and adipocyte-specific Fas knockout mice are partly protected from high fat diet-induced insulin resistance (11, 19) implicating a role for Fas in the pathogenesis of obesity-associated insulin resistance. While the underlying mechanism remained incompletely understood, livers of adipocyte-specific Fas-KO mice had lower levels of total ceramides, which are potentially metabolized from NEFA delivered to the liver from adipose tissue. Thus, in the present study we hypothesized that in adipocytes Fas activation can, independently of its pro-apoptotic effects, directly induce the hydrolysis of triglycerides (i.e., increase basal lipolysis), and set-up to investigate the intracellular signaling pathway(s) involved.

Materials and Methods

Cell Culture

3T3-L1 adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Basel Switzerland) containing 25 mM glucose (high glucose), supplemented with 10% fetal calf serum (FCS, Socochim SA, Lausanne) and antibiotics (Invitrogen). 48 hours after reaching confluence (day 0, D0), cells were treated with a mixture of 500 μ M methylisobutylxanthine, 1 μ M dexamethasone, 1.7 μ M insulin (all from Sigma, Buchs, Switzerland) and 1 μ M rosiglitazone (Alexis

Biochemicals) to induce differentiation. Two days later (D2) the medium was changed to high glucose culture medium containing insulin (0.5 μ M). Another 2 days later (D4), the medium was replaced by culture medium without insulin. The culture medium was replaced every other day and changed to culture medium containing 5.5 mM glucose (low glucose) after 4 days (D8). Cells were kept at least 2 days on low glucose before experiments were performed. Membrane-bound Fas ligand (Upstate, Lake Placid, NY, USA) was added to low glucose serum-free medium as indicated. For pretreatment experiments with rosiglitazone (Enzo Life Science, Lausen, Switzerland), mature adipocytes were incubated for 48 hours with 5 μ M of the compound in low glucose medium. Thereafter, FasL together with rosiglitazone was applied for another 6 or 12 hours.

For siRNA-mediated ERK1/2 knock down, mature 3T3-L1 adipocytes cells were treated with siRNA (target sequences for ERK1/2: 5' ACAAGCGCATCACAGTAGA 3', 5' GAACCCTAAGAGAGATAAA 3'; scrambled sequences for ERK1/2: 5' GCAACCGAACGGAAACATT 3', 5' GAACAAAGTAACGGTAACA 3') in a transfection mixture containing Lipofectamin 2000 (Invitrogen) in culture medium (low glucose, without FCS, without p/s) according to the manufacturer's instructions.

Western blotting

Adipocytes were lysed in ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 1mM sodium vanadate, 1 mM NaF, 10 mM sodium β -glycerophosphate, 100 nM okadaic acid, 0.2 mM PMSF and a 1:1000 dilution of protease inhibitor cocktail (Sigma). Protein concentration was determined using a BCA assay (Pierce, Rockford, IL, USA). Equal amounts of protein were resolved by lithium dodecyl sulfate (LDS)-PAGE (4-12% gel; NuPAGE, Invitrogen, Basel, Switzerland) and electro-transferred

onto nitrocellulose membranes (0.2 μ m, BioRad, Reinach, Switzerland). Protein content on membranes was checked by Ponceau S staining. Blots were blocked in tris-buffered saline containing 0.1% Tween (TBS-T) supplemented with 5% non fat dry milk. Primary antibody was applied in the same buffer in a dilution of 1:1000, secondary antibody in a dilution of 1:5000. Primary antibodies were purchased either from Cell Signaling Technology (phospho-p44/42 and total MAPKs, phospho-(Ser/Thr) PKA substrate, phospho-Ser 563 and total HSL, ATGL), MBL International, Woburn, MA, USA, (perilipin A), Millipore (Actin, Fas (CD95)), Vala Sciences (phospho-Perilipin Ser 522) or Santa Cruz Biotechnology (PPAR γ ₂, C/EBP α , pCaMKII α (Thr 286)). Signal was generated based on chemoluminescence and detected with the Fuji LAS-3000 image reader.

Measurement of lipolysis

Cells were incubated in DMEM without FCS and treated with 2 ng/ml FasL or 1 μ M isoproterenol in the presence or absence of 50 μ M of the MEK-inhibitor U0126 (Sigma), of 50 μ M of the intracellular calcium chelator BAPTA/AM (Calbiochem) or of 50 μ M of CaMKII-inhibitor KN62 (Sigma) as indicated. Inhibitors or BAPTA/AM were added to the cells at the same time as FasL. Thereafter, cells were washed in PBS and NEFA and glycerol were collected in Krebs-Ringer-HEPES buffer supplemented with 0.1% fatty acid free BSA for one hour. NEFAs in the supernatant were measured with the NEFA kit from Wako (Neuss, Germany) and glycerol with the free glycerol reagent from Sigma. Fractional re-esterification was calculated as previously described (20).

Data analysis

Data are presented as means \pm SEM and were analyzed by a one sample *t* test or analysis of variance (ANOVA) with a Newman-Keuls multiple comparison test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Results

Fas activation induces lipolysis in 3T3-L1 adipocytes

The Fas receptor (CD95) is expressed in 3T3-L1 preadipocytes, decreases during adipocyte differentiation but is still clearly expressed in mature adipocytes (Fig. 1). Expression levels of the adipocyte specific proteins PPAR γ_2 , C/EBP α and perilipin reflect the respective stage of differentiation. Treatment of differentiated 3T3-L1 adipocytes with 2 ng/ml FasL for 12 hours significantly increased lipolysis (Fig. 2), consistent with our earlier observation (8), and without affecting their viability as assessed by TUNEL assay (supplemental Fig. 1) and MTT determination (11). Fractional re-esterification did not decrease upon FasL incubation (basal: 68.0 \pm 2.6%, 6h FasL: 64.0 \pm 6.0%, 12h FasL: 63.6 \pm 5.5%, *p*=0.8). Such data suggest that Fas activation increases lipolysis rather by increased triglyceride hydrolysis than decreased re-esterification. Shorter incubation periods with FasL (\leq 6 hours) and lower concentrations of FasL did not increase lipolysis to a significant degree (supplemental Fig. 2). Moreover, blunted Fas-stimulated lipolysis in Fas depleted 3T3-L1 adipocytes suggests that membrane-bound FasL signals via the Fas receptor (supplemental Fig. 3).

Fas activation increases phosphorylation of HSL

Beta adrenergic receptor agonists such as catecholamines stimulate lipolysis via adenylate cyclase-dependent activation of PKA and consecutive activation of HSL, perilipin 1 and ATGL. Inhibiting such effect, insulin activates phosphodiesterase 3, which converts cAMP to 5'-AMP, thereby diminishing cAMP-mediate PKA activity, which results in inhibition of lipolysis. In order to examine whether Fas-mediated lipolysis comprise activation of PKA, phosphorylation of PKA substrates was determined in 3T3-L1 adipocytes. As expected, the $\beta_{1,2}$ -receptor agonist isoproterenol increased phosphorylation of PKA substrates significantly. In contrast, treatment with FasL had no effect on the abundance of phosphorylated PKA substrates (Fig. 3A). However, even though not detected by the PKA substrate antibody, incubation of 3T3-L1 adipocytes with FasL for 6 and 12 hours significantly increased phosphorylation of HSL at Ser563 (Fig. 3B), while it had no effect on total HSL protein levels (supplemental Fig. 4) and phosphorylation of perilipin (Fig. 3B). In addition, ATGL protein levels were slightly but not significantly up regulated upon Fas incubation (Fig. 3C). Thus, Fas-mediated lipolysis may depend on activation of HSL and/or ATGL.

Fas-mediated lipolysis is ERK-dependent

An alternative signaling pathway to activate lipolysis in adipocytes involves the p44/42 MAP kinases (ERK1/2), as was shown for $\text{TNF}\alpha$ (21). Since Fas belongs to the tumor necrosis factor receptor superfamily, we postulated that FasL-induced lipolysis is mediated via ERK1/2 activation. Incubation of mature 3T3-L1 adipocytes with 2 ng/ml FasL increased phosphorylation of ERK1/2 significantly after 6 and 12 hours whereas total protein concentration of ERK1/2 was not affected (Fig. 4A). To exclude the possibility that the effects of FasL treatment were due to a FasL-

mediated increase in $\text{TNF}\alpha$ secretion and, thus, to a paracrine regulatory loop mediated by this cytokine, $\text{TNF}\alpha$ concentration was determined in the supernatant. As depicted (supplemental Fig. 5), incubation with 2 ng/ml FasL for 12 hours did not lead to increased $\text{TNF}\alpha$ secretion to the medium. We therefore concluded that Fas-mediated ERK1/2 activation was independent of $\text{TNF}\alpha$.

To assess whether Fas-induced lipolysis is dependent on ERK1/2 activation, we incubated 3T3-L1 cells in presence or absence of the MEK1/2 inhibitor U0126. Inhibition of the MAP kinase pathway with U0126 (50 μM) completely blocked Fas induced ERK1/2 phosphorylation and lipolysis (Fig. 4B). To further strengthen a role of the p44/42 MAP kinase pathway in Fas-mediated lipolysis, 3T3-L1 adipocytes were treated with targeted or scrambled siRNA against ERK1/2, respectively. Compared to scrambled siRNA control, siRNA-targeted towards ERK1/2 decreased ERK1/2 protein by about 50%, an effect that was associated with a significant blunting of FasL-stimulated lipolysis (Fig. 4C). Thus, Fas-stimulated lipolysis is dependent on ERK1/2 activation.

To further corroborate a Fas-ERK1/2-lipolysis pathway in adipocytes, we tested whether $\text{PPAR}\gamma$ agonists such as thiazolidinediones (TZDs), which were demonstrated to inhibit whole body lipolysis in patients with type 2 diabetes (22), can inhibit Fas-induced lipolysis and if so, whether activation of ERK1/2 was also diminished. As shown in Fig. 4D, FasL-stimulated ERK1/2 phosphorylation was reduced in the presence of rosiglitazone without affecting total ERK1/2 protein content. Correspondingly, rosiglitazone also reduced FasL-mediated lipolysis (Fig. 4D).

Fas-mediated lipolysis is CaMKII-dependent

CaMKII was previously shown to mediate magnolol-triggered lipolysis in sterol ester-loaded 3T3-L1 preadipocytes in an ERK1/2 dependent fashion (23). We therefore postulated that FasL-induced lipolysis may be dependent on intracellular changes in calcium levels since it is well accepted that Fas activation can increase intracellular calcium levels (24). Indeed, preincubation of 3T3-L1 adipocytes with the intracellular calcium chelator BAPTA/AM prevented both FasL-induced ERK-activation and lipolysis (Fig. 5A), suggesting that both effects of Fas activation are dependent on an intracellular calcium rise. In response to the latter the developing calcium/calmodulin complex may activate CaMKII leading to intramolecular autophosphorylation at several sites including Thr286, Thr305 and Thr306 (25). As depicted in Fig. 5B, incubation of mature 3T3-L1 adipocytes with 2 ng/ml FasL increased phosphorylation of CaMKII at Thr286 significantly after 6 and 12 hours. Moreover, pre-treatment with the CaMKII inhibitor KN62 reduced FasL-mediated ERK1/2 activation as well as lipolysis (Fig. 5C). These data strongly suggest that Ca-mediated activation of CaMKII is a proximal signaling response to Fas activation, which is propagated further downstream via ERK1/2 to induce basal lipolysis.

Discussion

In obesity, higher basal lipolysis rate resulting in increased release of NEFAs into the circulation contributes to the development of hepatic and total body insulin resistance (26). Several factors contribute to such increase in lipolysis. First, obesity is associated with a persistent low grade inflammation of adipose tissue as manifested by an increased production and secretion of pro-inflammatory cytokines such as $\text{TNF}\alpha$ and IL-6 (27). The latter in turn can directly stimulate adipocyte

lipolysis even as isolated factors (21, 28, 29). Second, hypertrophic adipocytes are characterized by an elevated rate of basal lipolysis (30), which might be at least partly mediated by self-production of inflammatory cytokines acting in an autocrine manner, possibly as a self-protective cellular mechanism against excessive cellular over-growth. Third, since insulin is the major anti-lipolytic hormone, insulin resistance at the adipocyte level results in increased lipolysis, creating a vicious cycle between hypertrophy, inflammation, lipolysis, and insulin resistance. Fas may be a key component of such dys-regulation: Fas is activated by FasL, which can be produced by inflammatory cells infiltrating adipose tissue in obesity. Moreover, expression of Fas is increased in adipose tissue of obese humans and in isolated adipocytes of obese and diabetic mice, and intriguingly, its protein expression correlates with adipocyte size and is therefore increased in hypertrophic adipocytes (11).

In agreement with such notion, we show herein that chronic stimulation of the death receptor Fas induced lipolysis in 3T3-L1 adipocytes. Importantly, this effect occurred under conditions that did not induce apoptosis (11). Like $\text{TNF}\alpha$, FasL stimulates lipolysis through activation of the ERK1/2 MAP kinases since Fas activation lead to increased phosphorylation of ERK1/2 and pre-treatment with the MEK1/2 inhibitor U0126 or siRNA-mediated downregulation of ERK1/2 blocked Fas-induced lipolysis. In the case of $\text{TNF}\alpha$ it was proposed that ERK1/2-dependent down-regulation of the lipid droplet coating protein perilipin is responsible for the increase in lipolysis (21). We also observed a down-regulation of perilipin in cells treated with FasL for 12 hours. However, in contrast to $\text{TNF}\alpha$ such effect was not mediated by ERK1/2, since FasL-induced decrease in perilipin expression was not prevented by ERK-inhibition (supplemental Fig. 6). Moreover, 6 hours of FasL incubation did not decrease perilipin protein content (data not shown) but increased FFA and glycerol

release. All these results suggest that Fas-mediated lipolysis is independent of a decrease in perilipin expression.

Herein, we present evidence for calcium-triggered activation of ERK in Fas activation-mediated lipolysis. Fas activation in cells was previously shown to raise intracellular free Ca^{2+} levels (24). Similarly, increased intracellular Ca^{2+} levels induced by endoplasmic reticulum (ER) stress were shown to induce lipolysis in adipocytes ERK-dependently (31). Hence, Fas-induced ERK activation may be mediated by ER stress-triggered Ca^{2+} release. However, as shown in supplemental Figure 7, FasL stimulation of 3T3-L1 adipocytes did not provoke ER stress. Moreover, the extracellular Ca^{2+} -chelator EDTA blunted Fas-induced ERK activation and lipolysis similar to the intracellular chelator BAPTA (supplemental Fig. 8). Such data suggest that extracellular Ca^{2+} -influx rather than Ca^{2+} -release from ER is involved in Fas induced lipolysis. Increased intracellular Ca^{2+} is bound by the calcium-binding protein calmodulin (CaM) forming a complex. The latter then binds to and thereby activates CaMKII. Activation of CaMKII by the Ca^{2+} /CaM complex allows intramolecular autophosphorylation at several sites including Thr286. This generates calcium-independent activity that persists after dissociation of calcium/calmodulin allowing transient calcium elevation to promote prolonged kinase activation (25). We found that FasL treatment increased phosphorylation at Thr286 of CaMKII. Moreover, Fas activation-induced lipolysis was prevented in the presence of the CaMKII-inhibitor as well as of the Ca^{2+} -chelator BAPTA. Thus, we postulate that Fas activation in adipocytes increases intracellular Ca^{2+} levels leading to activation of CaMKII, which in turn activates ERK1/2 and, thus, lipolysis. Accordingly, it was previously reported that *trans*-10, *cis*-12 conjugated linoleic acid-induced ERK1/2 activation in adipocyte is dependent on a rise in intracellular free Ca^{2+} levels and consecutive activation of CaMKII (32). Unfortunately, lipolysis was not addressed in

this study. The data presented herein may suggest a novel pathway for lipolysis in adipocytes via CaMKII-dependent activation of ERK. Interestingly, such pathway may be very ancient and evolutionarily preserved since CaMKII-mediated release of free fatty acids was recently demonstrated to play a role in pheromone biosynthesis in insects (33).

Interestingly, the TZD rosiglitazone reduced both Fas-mediated ERK1/2 phosphorylation and lipolysis. PPAR γ agonists have profound effects on adipocyte metabolism and were shown to improve insulin sensitivity in patients with type 2 diabetes. Moreover, in a recent paper, treatment with another TZD, pioglitazone, was able to reduce whole body lipolysis in type 2 diabetic patients (22). We previously described a potential role for Fas activation in obesity-associated insulin resistance (11) and show herein that Fas activation leads to increased lipolysis. Thus, our experiments may point to a role of TZDs in counteracting Fas-induced metabolic changes in adipocytes and further underscore the importance of ERK-activation for FasL-induced lipolysis. Similarly, TZDs were previously found to antagonize the effects of TNF α on adipocytes (34).

We recently showed that treatment of 3T3-L1 adipocytes with 2ng/ml FasL for 12 hours reduced protein levels of Akt/PKB (protein kinase B) (19). Moreover, a recent publication reported that deletion of rictor, an essential component of the Akt kinase mammalian target of rapamycin complex 2 (mTORC2), increases basal lipolysis as well as phosphorylation of HSL at Ser563 in adipocytes (35). Hence, it is conceivable that Fas mediated reduction in Akt protein levels contributed to FasL-induced lipolysis, potentially via increased phosphorylation of HSL at serine residue 563. Moreover, ERK1/2-mediated phosphorylation of HSL at Ser600 (36) might contribute to Fas-induced lipolysis. However, such corresponding anti-phospho-HSLSer600 antibody is not commercially available and, thus, not available to us for

evaluating a potential role of HSLSer600 phosphorylation in this study. Besides activation of HSL, we cannot exclude the involvement of ATGL to Fas activation-induced lipolysis since ATGL protein levels were slight albeit not significantly increased after FasL treatment.

In summary, activation of the Fas receptor alters the metabolism of adipocytes and leads to ERK1/2-mediated lipolysis, which may be triggered by Fas-induced increase in intracellular calcium levels and hence, autophosphorylation of the Ca^{2+} /calmodulin-dependent protein kinases II. Thus, our findings suggest an important role of the Fas receptor in the development of adipose tissue dys-function in the context of obesity.

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Figure Legends

Fig. 1 The Fas receptor is expressed in 3T3-L1 cells. Lysates of 3T3-L1 preadipocytes (PreAC), differentiating (day (D) 1, 2, 4 and 6) and mature adipocytes (AC) were resolved by LDS-PAGE and immunoblotted with an antibody against the Fas receptor, PPAR γ ₂, C/EBP α and perilipin.

Fig. 2 Fas stimulation induces lipolysis in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with FasL (2 ng/ml) for indicated time periods. NEFA (**A**) and glycerol (**B**) concentrations were determined in the supernatant collected for 1 hour as described under “Experimental Procedures”. Results represent the means \pm SEM of six independent experiments. * $p < 0.05$.

Fig. 3 Fas activation increases phosphorylation of HSL. Fully differentiated 3T3-L1 adipocytes were treated with FasL (2 ng/ml) for indicated time periods or isoproterenol (1 μ M) for 30 min. Lysates were resolved by LDS-PAGE and immunoblotted with phospho-(Ser/Thr) PKA substrate (**A**), phospho-HSL (Ser563) and phospho-Perilipin (**B**) or ATGL (**C**) antibodies. Shown are representative blots and densitometry analyses of four to eight independent experiments * $p < 0.05$, ** $p < 0.01$.

Fig. 4 Fas-mediated lipolysis is ERK-dependent. (**A**) Fully differentiated 3T3-L1 adipocytes were treated with FasL (2 ng/ml) as indicated. Lysates were resolved by LDS-PAGE and immunoblotted with phosphorylated (Thr202/Tyr204) and total ERK1/2 antibodies. Shown are representative blots and quantification of seven independent experiments. (**B**) 3T3-L1 adipocytes were treated with 2 ng/ml FasL and

different concentrations of the MEK inhibitor U0126. Shown are representative blots in the upper panel. For the determination of lipolysis cells were incubated with FasL for 12 hours in the presence (white bars) or absence (black bars) of 50 μ M U0126 and glycerol release was determined. Results are the means \pm SEM of six independent experiments. (**C**, left panel) siRNA treated cells (scrambled (scr, black bars) or target (white bars)) were lysed 72 hours after transfection. Total cell lysates were resolved by LDS-PAGE and immunoblotted with total ERK1/2 and actin antibodies. Shown are representative blots (upper panel) and densitometry analyses of four independent experiments (lower panel). (**C**, right panel) Glycerol release was determined 72 hours after siRNA-mediated ERK1/2 depletion. Results are the means \pm SEM of six independent experiments. (**D**) Cells were incubated with or without 5 μ M rosiglitazone for 60 hours. During the last 6 or 12 hours of such incubation cells were treated with FasL. Left panel, total cell lysates were resolved by LDS-PAGE and immunoblotted with anti-phospho-ERK1/2 or anti-ERK1/2 antibodies. Shown are representative blots and quantification of four independent experiments. Right panel, glycerol release was determined. Results are the means \pm SEM of four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5 FasL-induced lipolysis is CaMKII dependent. (**A**) 3T3-L1 adipocytes were incubated with 2ng/ml FasL and different concentrations of BAPTA/AM for 12 hours. Left panel, the amount of phosphorylated and total ERK1/2 was analyzed in whole cell lysates by Western blot technique. Shown are representative blots and quantification of four independent experiments. Right panel, glycerol release was determined in adipocytes treated with or without 2ng/ml FasL and 50 μ M BAPTA/AM for 12 hours. Results are the means \pm SEM of three independent experiments. (**B**) 3T3-L1 adipocytes were treated with FasL (2 ng/ml) for indicated time periods.

Lysates were resolved by LDS-PAGE and immunoblotted with phospho-CaMKII and actin antibodies. Shown are representative blots and quantification of five independent experiments. **(C)** 3T3-L1 adipocytes were incubated with 2ng/ml FasL and different concentrations of the CaMKII inhibitor KN62 for 12 hours. Left panel, phosphorylated ERK1/2 was analyzed in whole cell lysates by Western blot technique. Shown are representative blots and quantification of four independent experiments. Right panel, glycerol release was determined in 3T3-L1 adipocytes treated with or without FasL and 10 μ M KN62. Results are the means \pm SEM of five independent experiments. * $p < 0.05$, *** $p < 0.001$.

Fig. 1

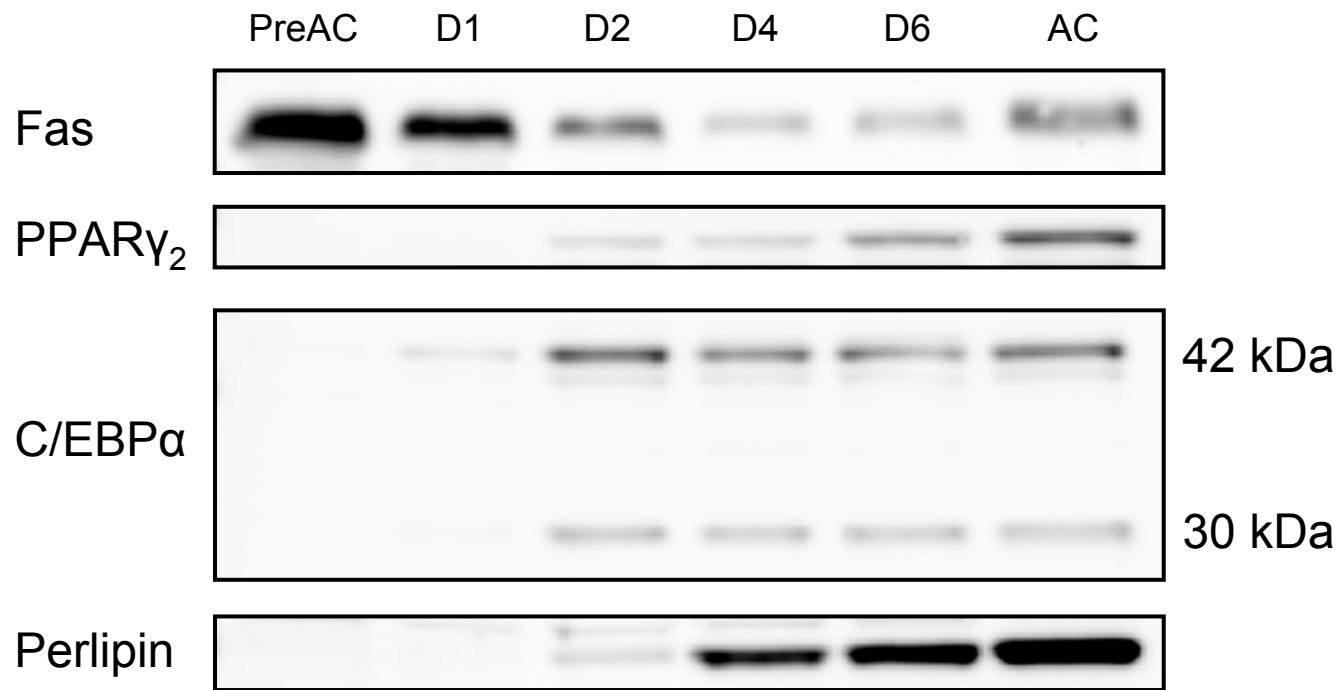


Fig. 2

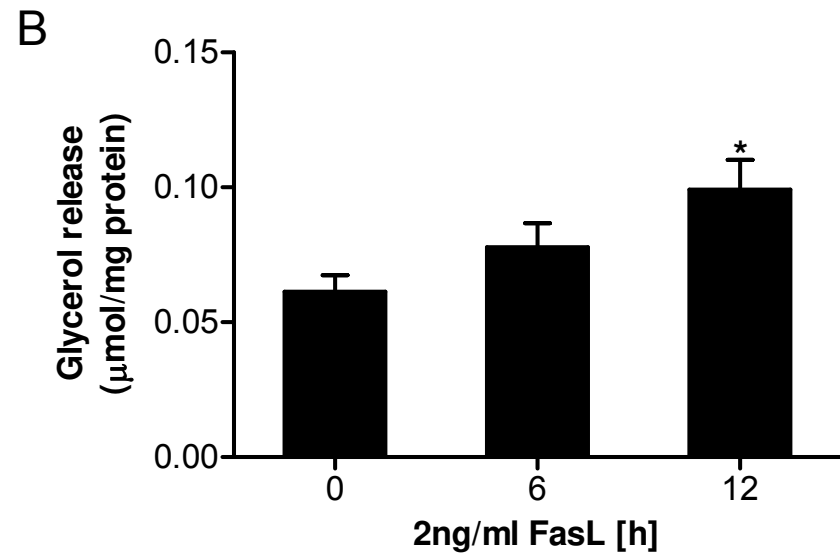
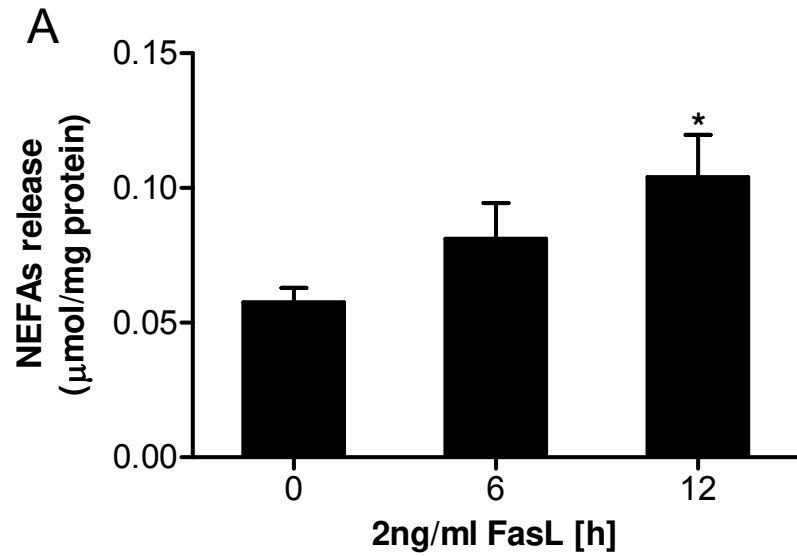


Fig. 3

A

MW

Co

FasL
6 h

FasL
12 h

Isop.

120
100
80
60
50
40
30

pPKA substrate

Actin

Phospho-PKA substrate
(relative to actin)

2.5
2.0
1.5
1.0
0.5
0.0

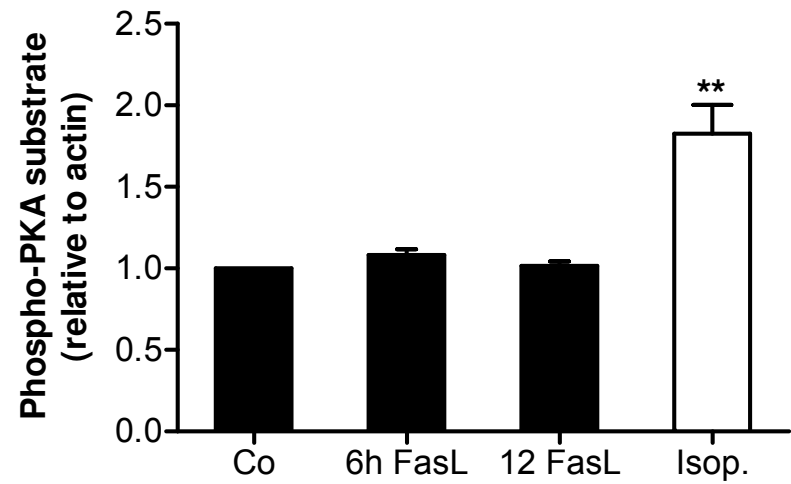
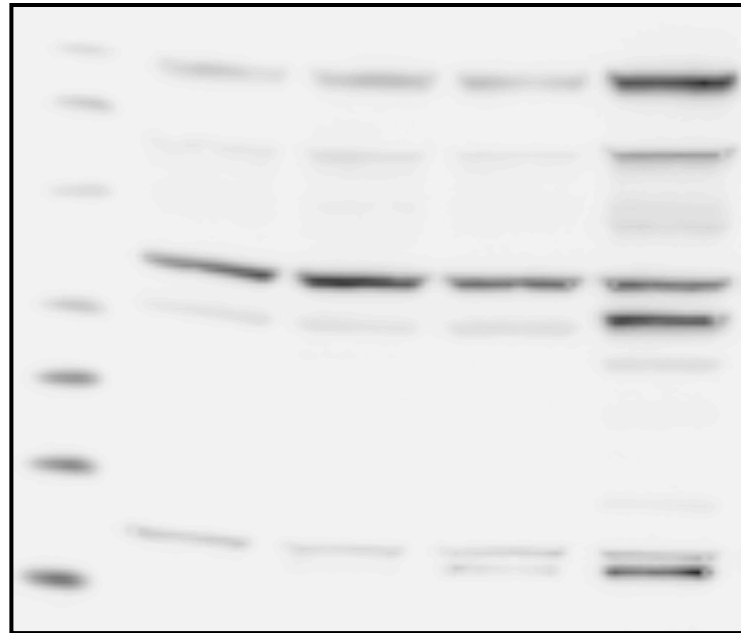
Co

6h FasL

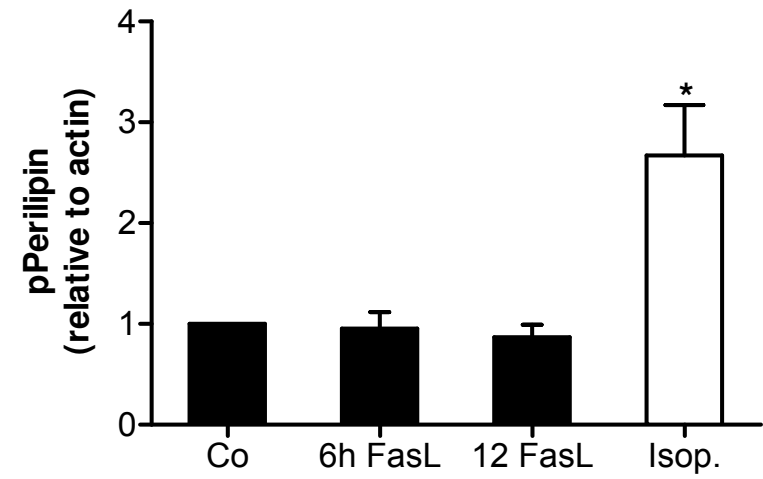
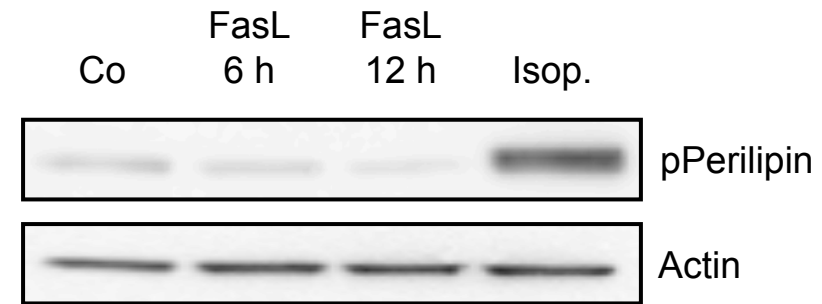
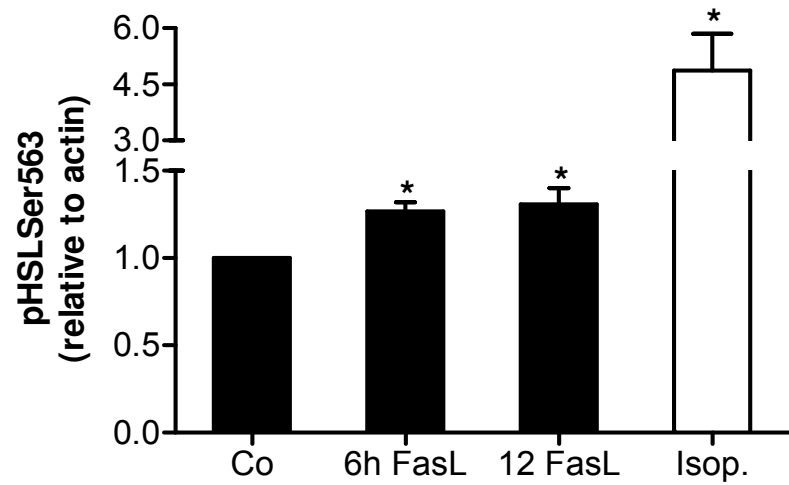
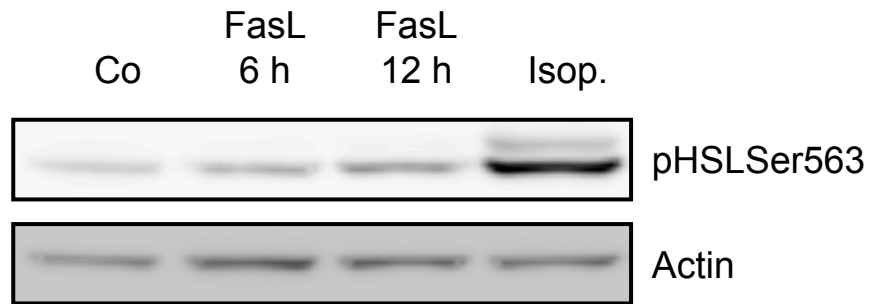
12 FasL

Isop.

**



B



C

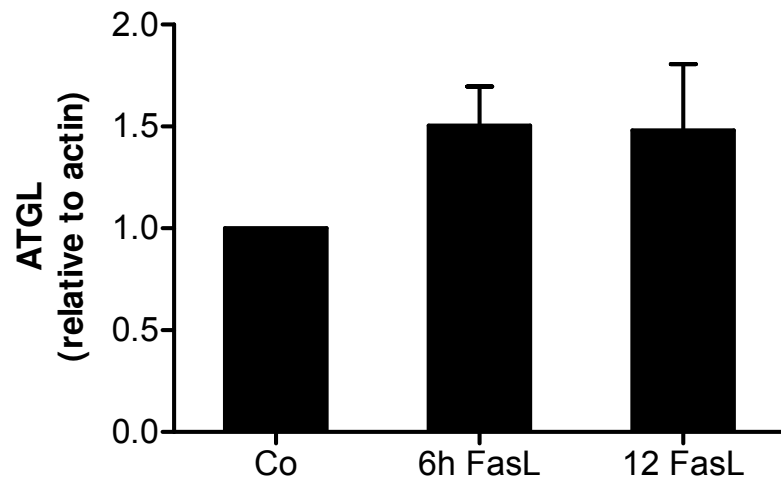
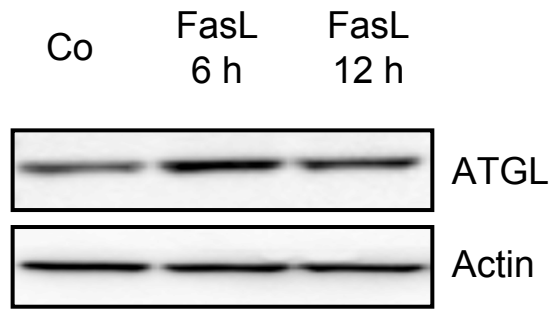
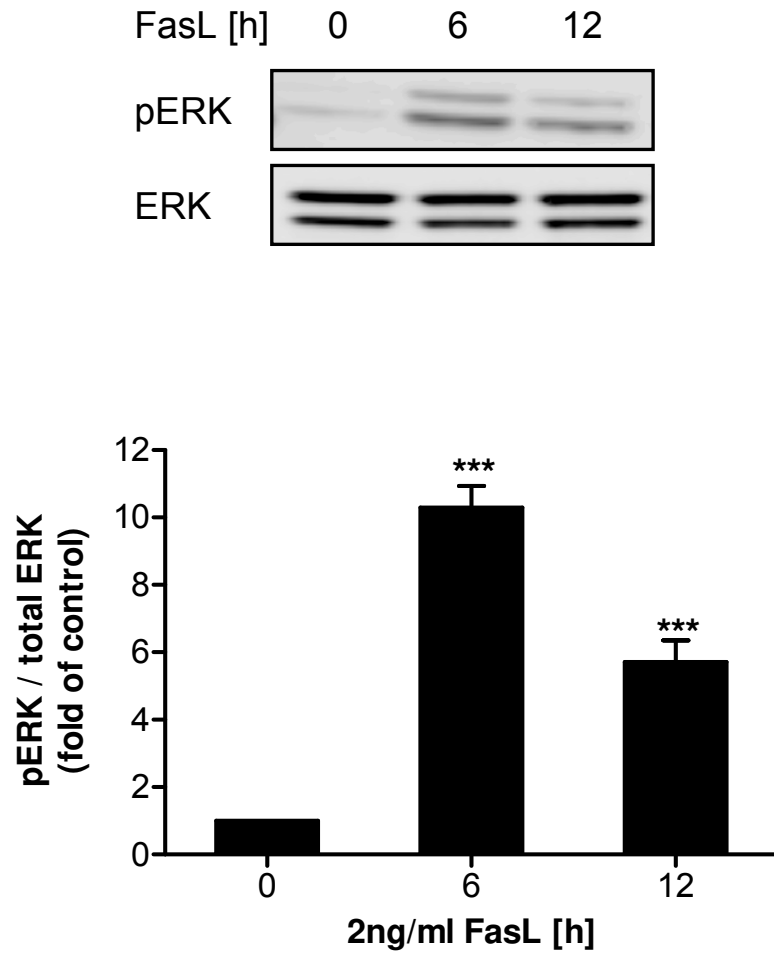
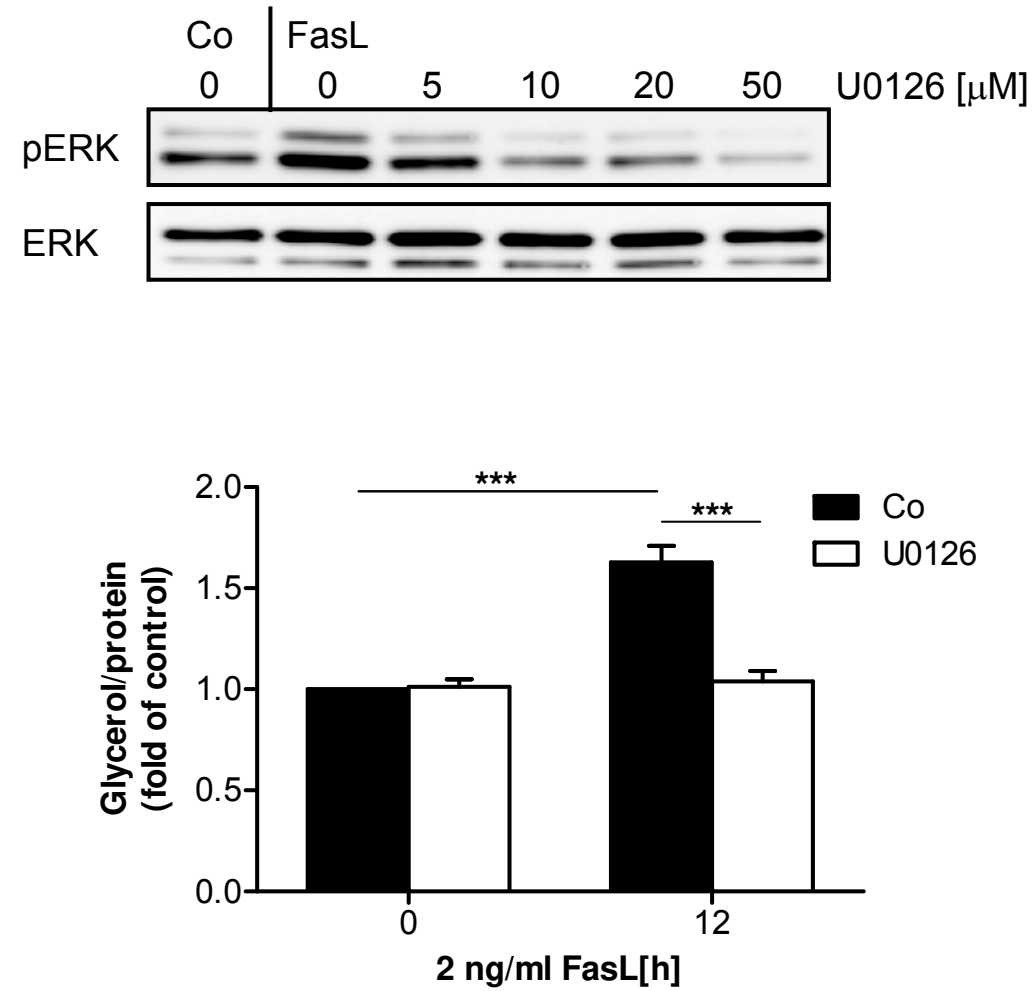


Fig. 4

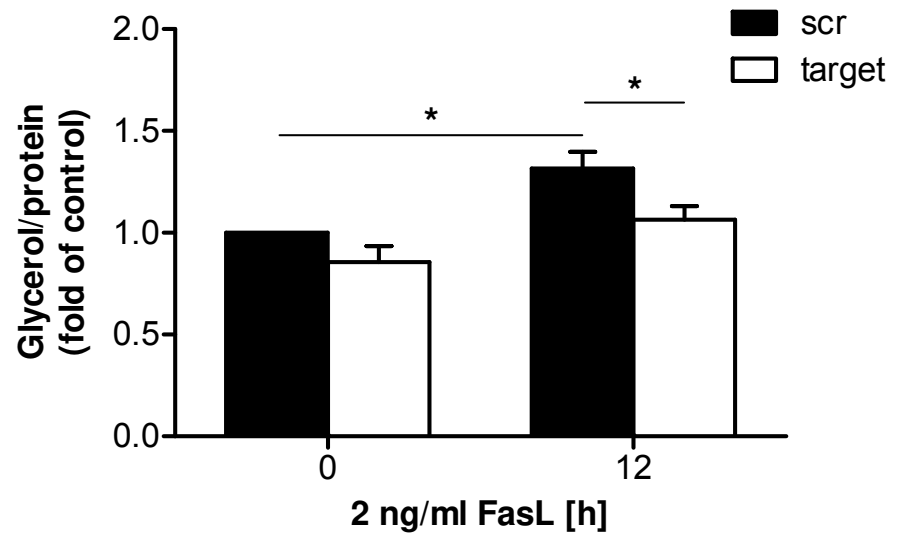
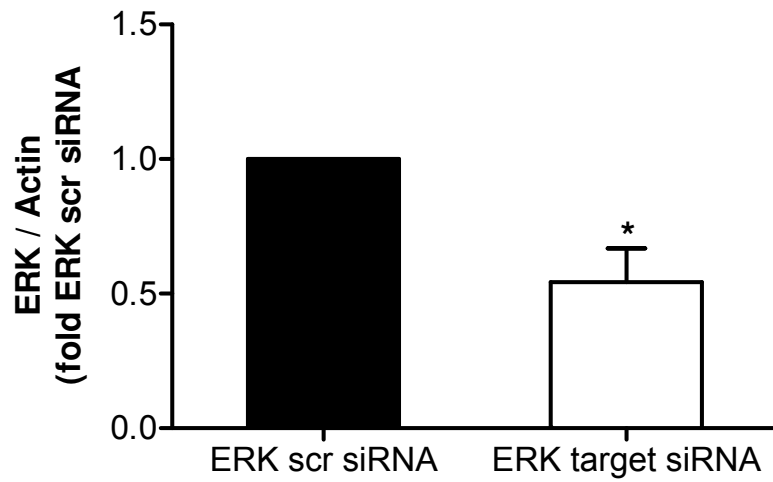
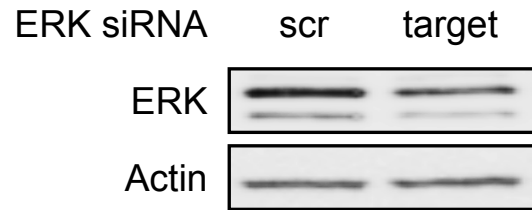
A



B



C



D

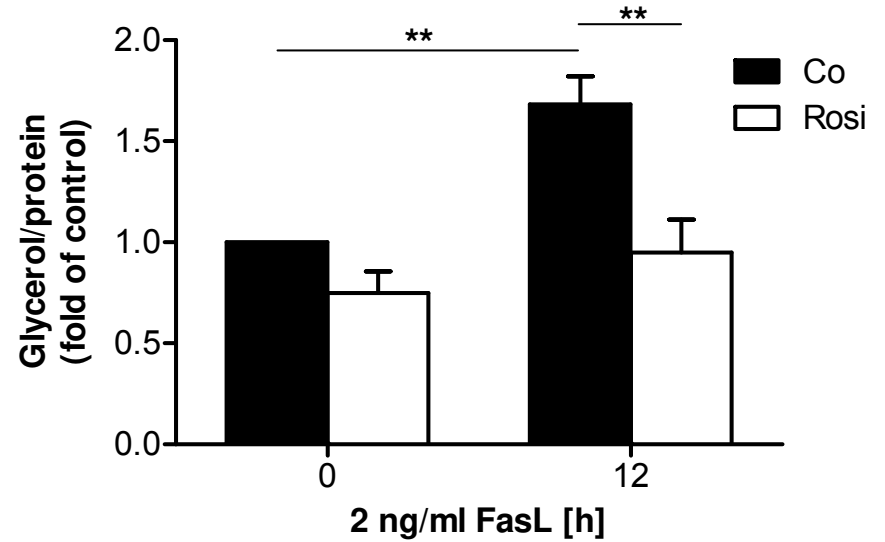
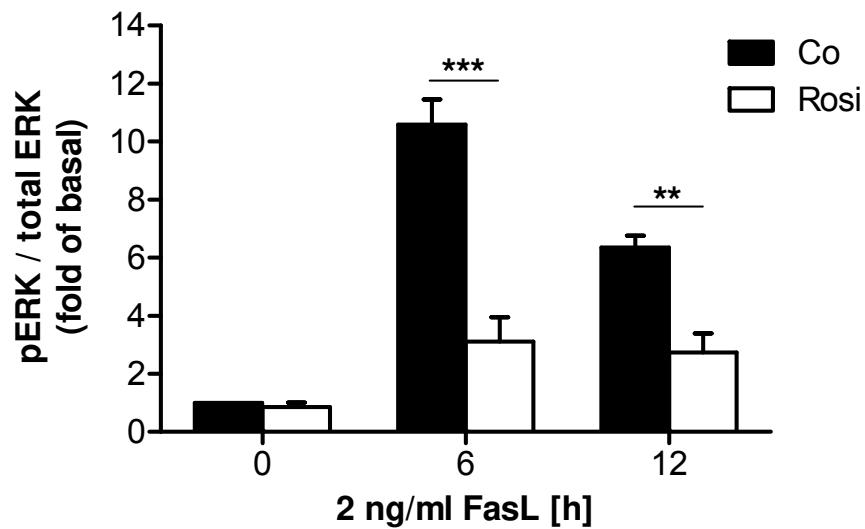
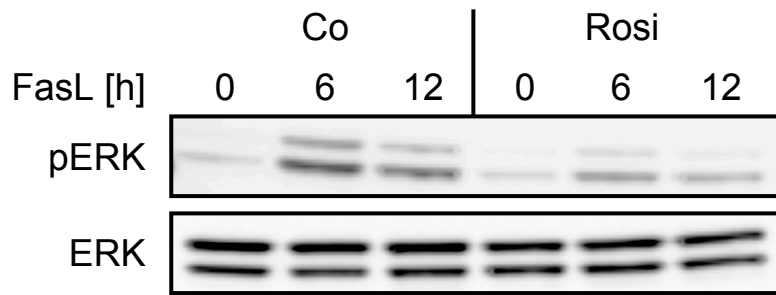
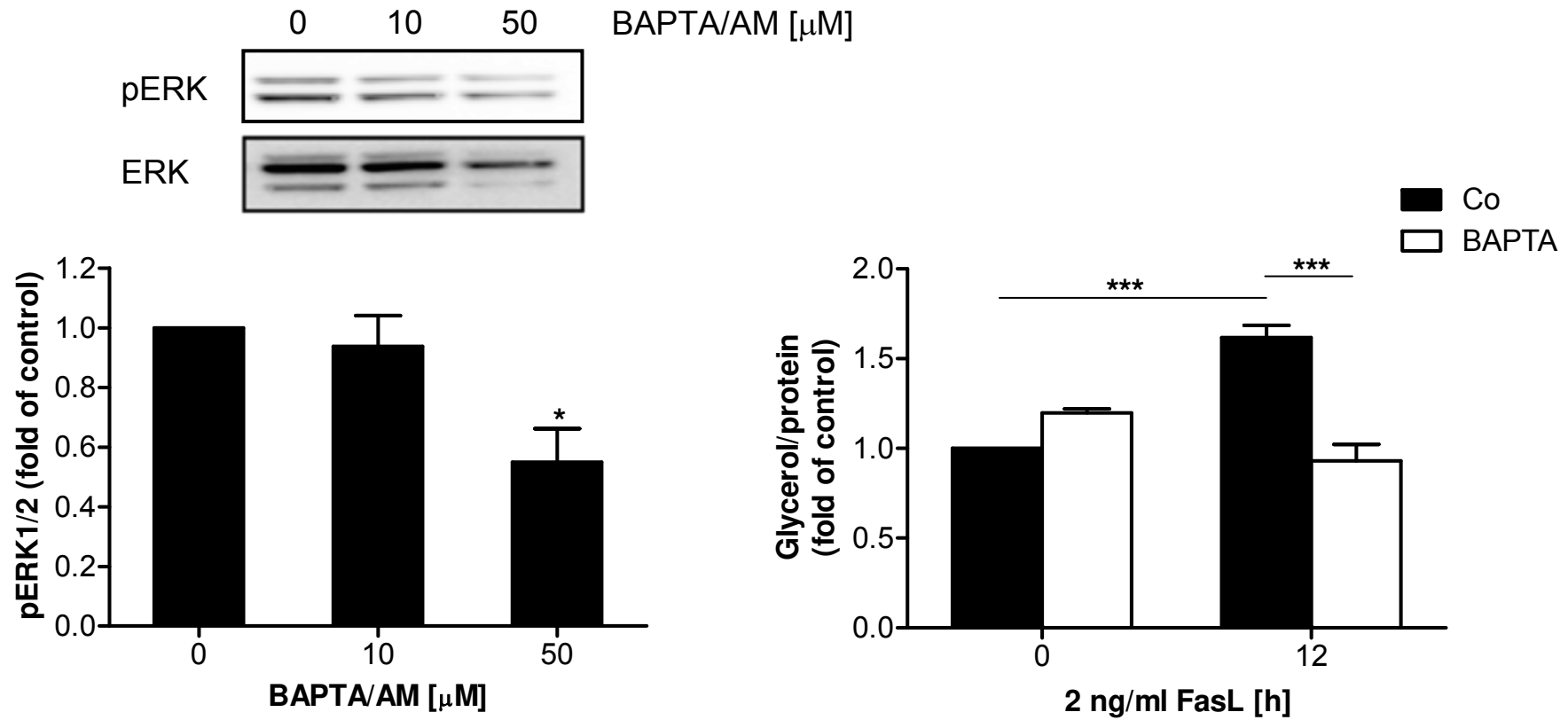
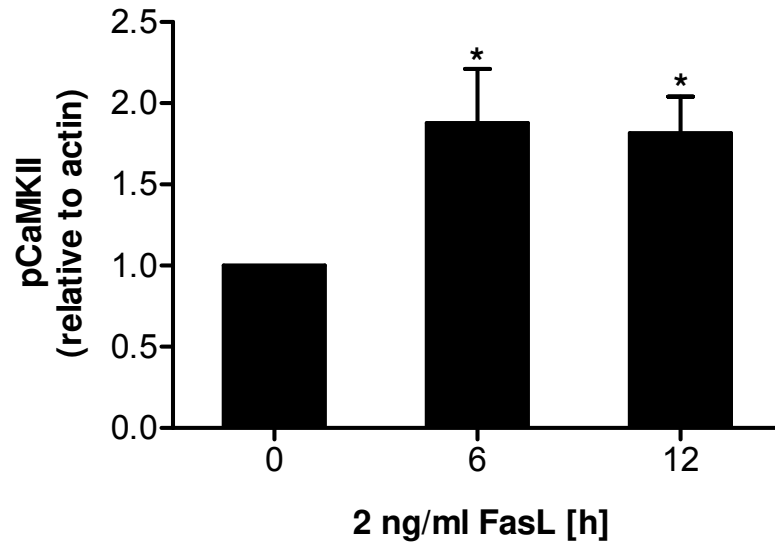
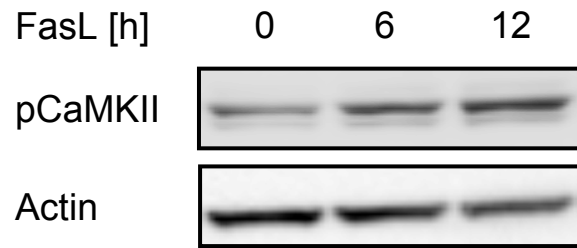


Fig. 5

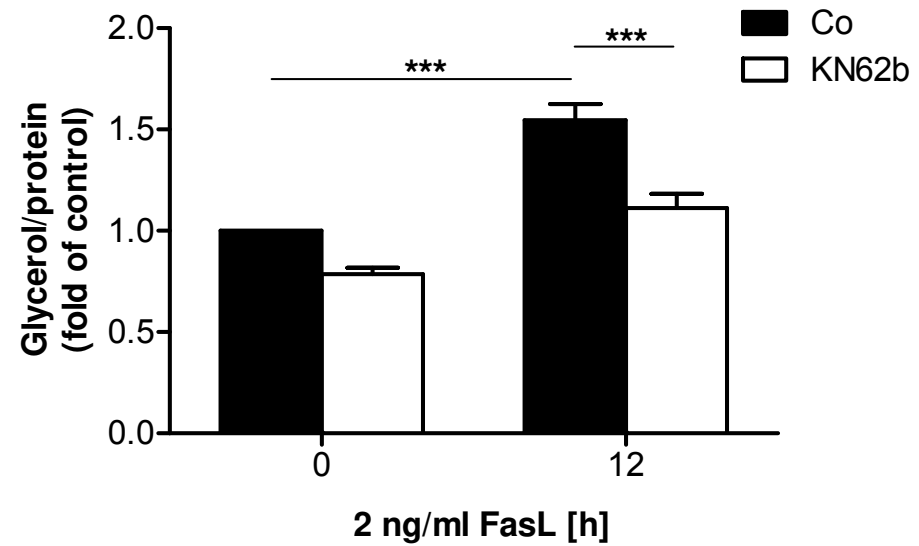
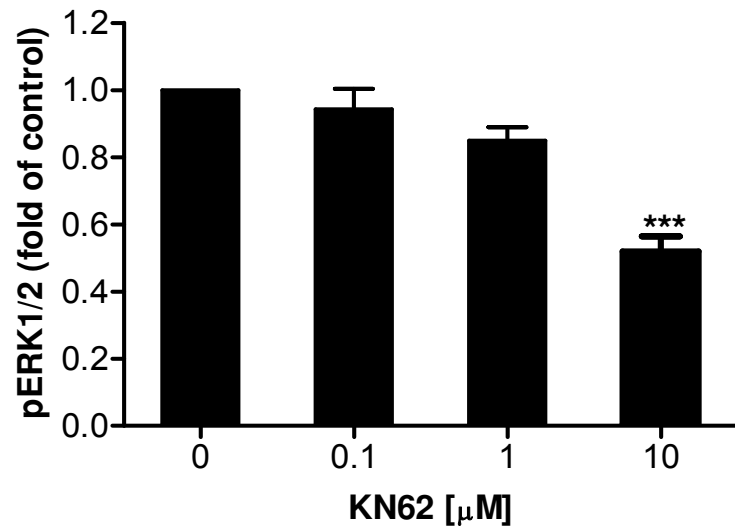
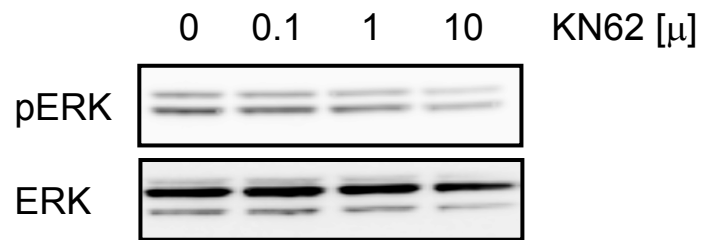
A

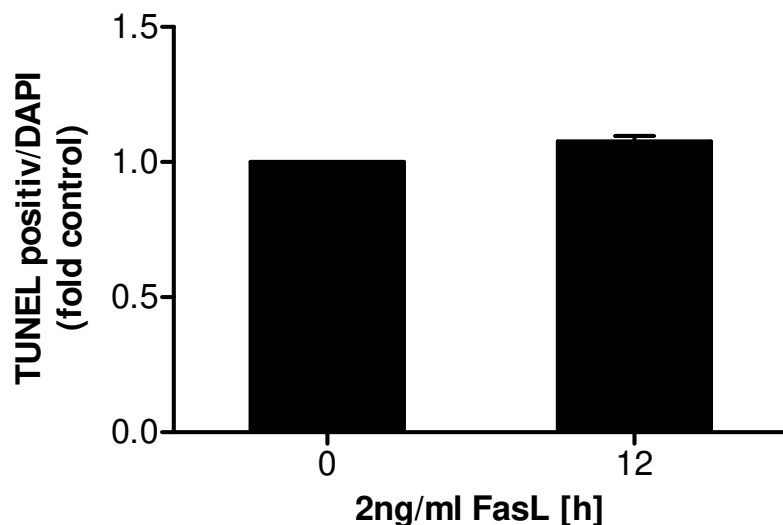


B



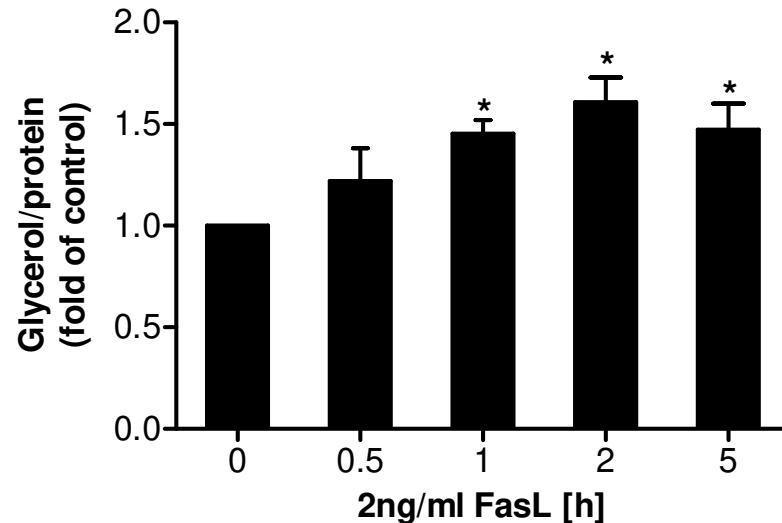
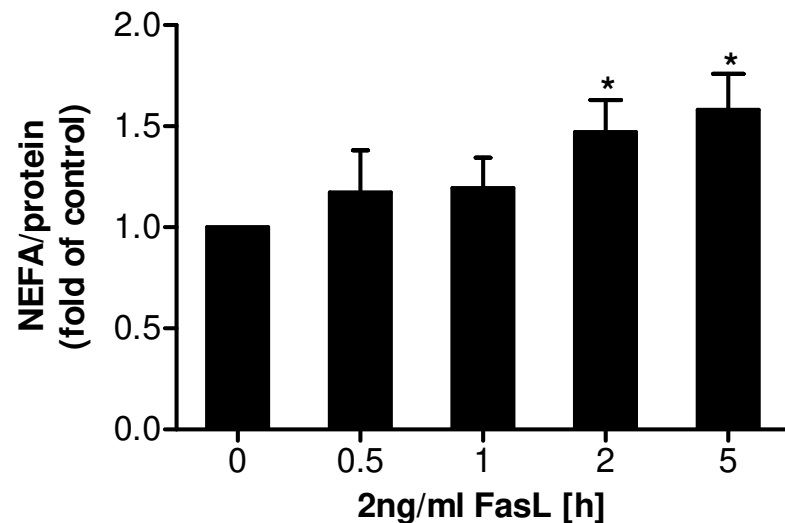
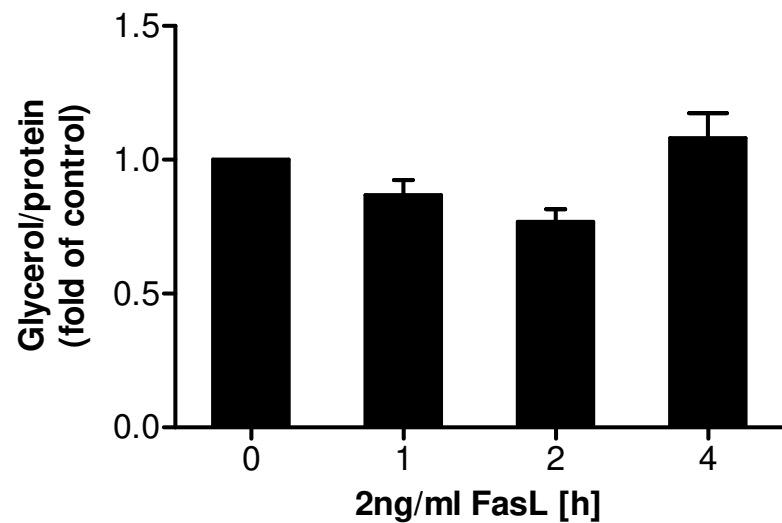
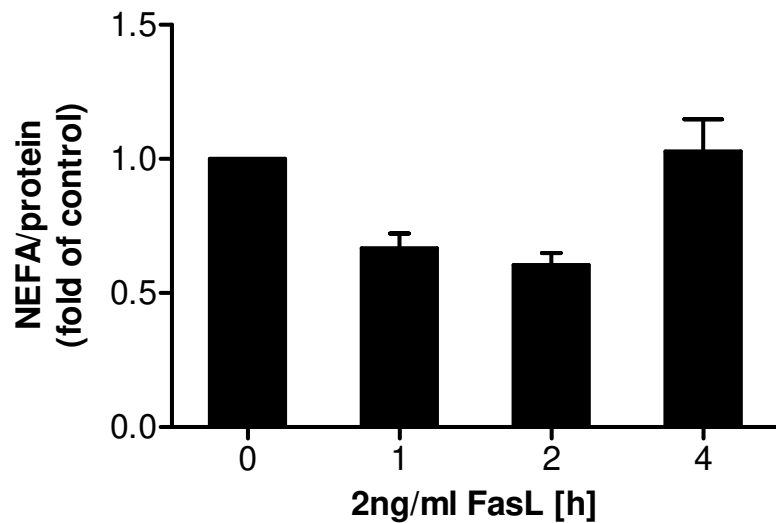
C





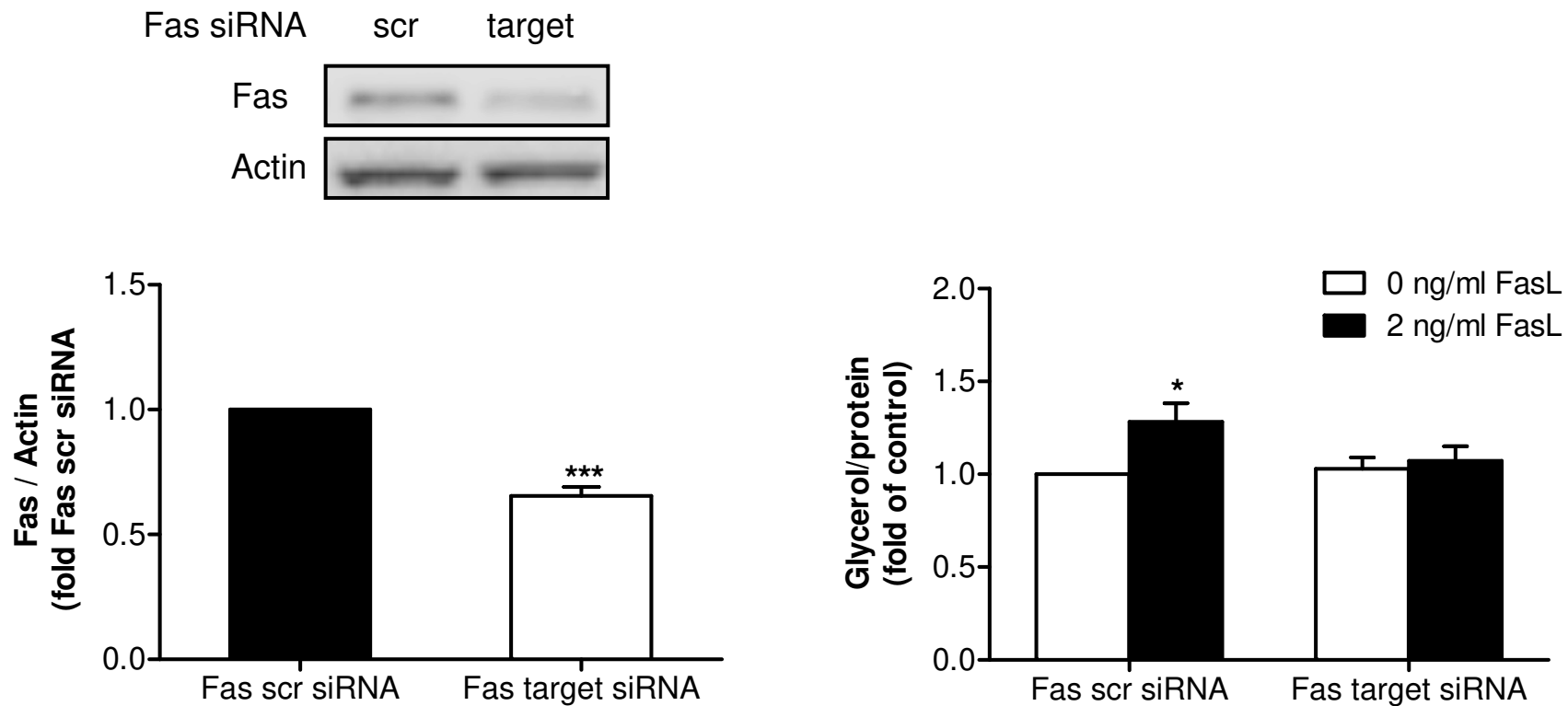
Supplemental Figure 1 Incubation with 2ng/ml FasL for 12 hours does not induce apoptosis in 3T3-L1 adipocytes

Mature 3T3-L1 adipocytes were incubated for 12 hours with or without 2ng/ml FasL. Thereafter, cells were stained with DAPI and TUNEL and the ratio of TUNEL-positive to DAPI positive cells was determined. Results represent the means \pm SEM of 3 independent experiments and are expressed relative to untreated cells



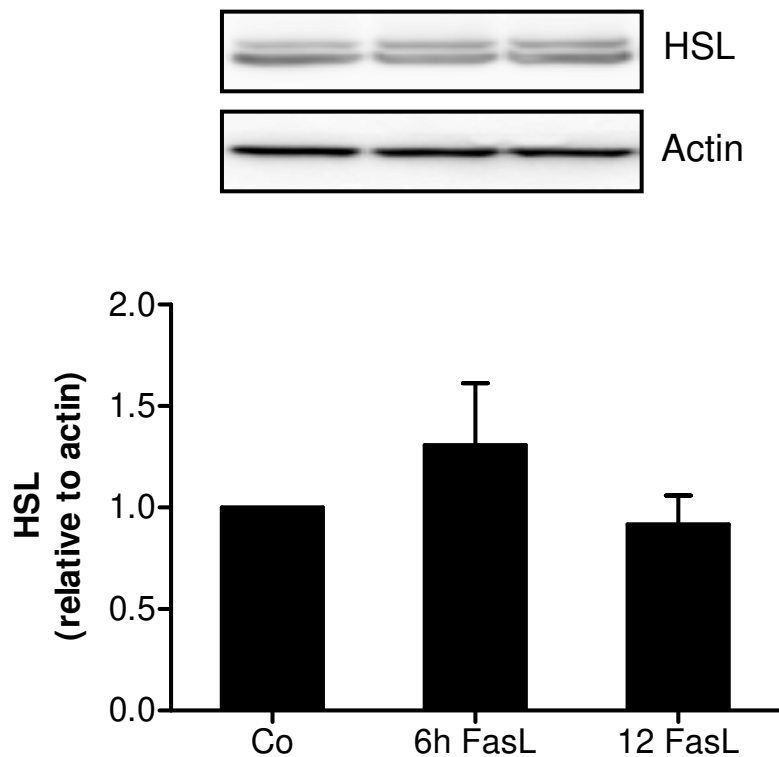
Supplemental Figure 2 Incubation with 2ng/ml FasL for less than 6 hours does not induce lipolysis in 3T3-L1 adipocytes

Fully differentiated 3T3-L1 adipocytes were treated with different concentrations of FasL for indicated time periods. NEFA and glycerol concentrations were determined in the supernatant collected for 1 hour. Results represent the means \pm SEM of four to five independent experiments. * $p < 0.05$



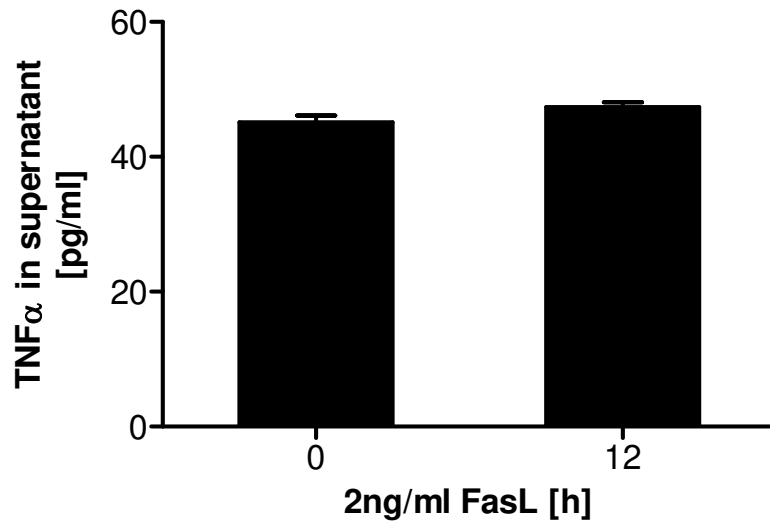
Supplemental Figure 3 Blunted FasL-stimulated lipolysis in Fas depleted 3T3-L1 adipocytes

Fas protein levels and glycerol release was determined 72 hours after siRNA-mediated Fas depletion. Results are the means \pm SEM of five to six experiments. * $p < 0.05$, *** $p < 0.001$.



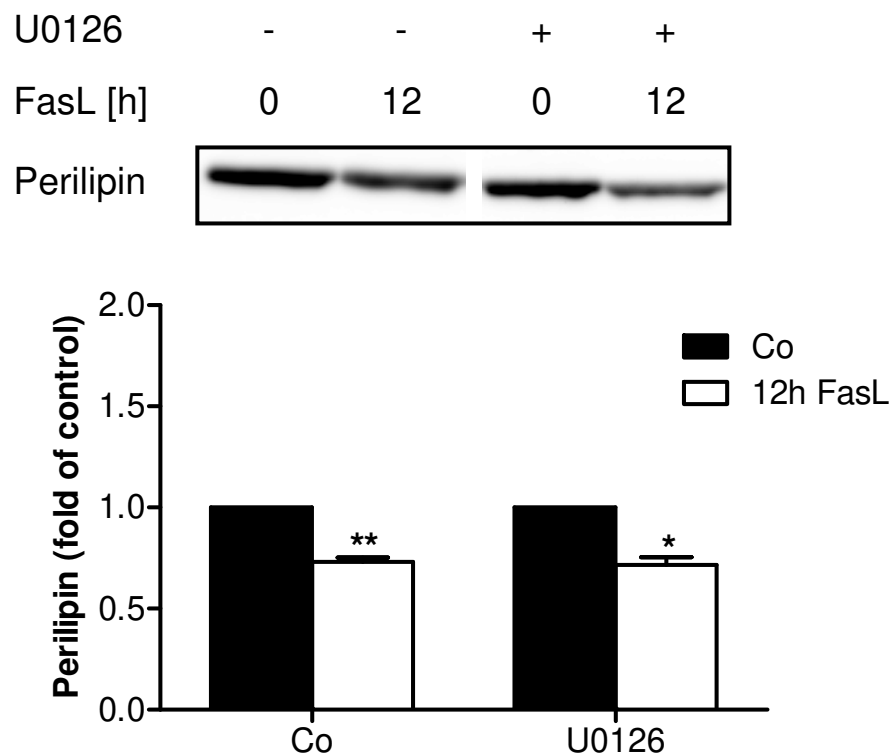
Supplemental Figure 4 Fas activation does not affect HSL protein levels

Fully differentiated 3T3-L1 adipocytes were treated with FasL (2 ng/ml) for indicated time periods. Lysates were resolved by LDS-PAGE and immunoblotted with HSL or actin antibodies. Shown are representative blots and densitometry analyses of five independent experiments.



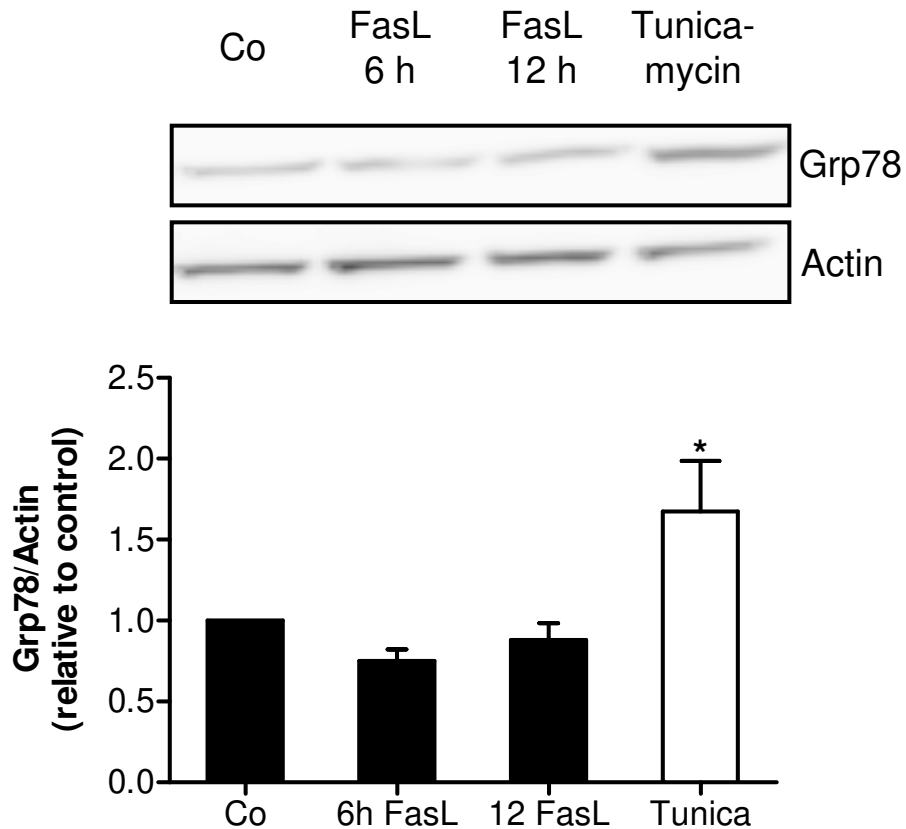
Supplemental Figure 5 Fas activation does not induce TNF α secretion

Adipocytes were incubated with or without 2ng/ml FasL for 12 hours. Cells were washed and incubated with KREBS buffer for another hour. Supernatant was collected and TNF α was determined. Results represent the means \pm SEM of 5 independent experiments.



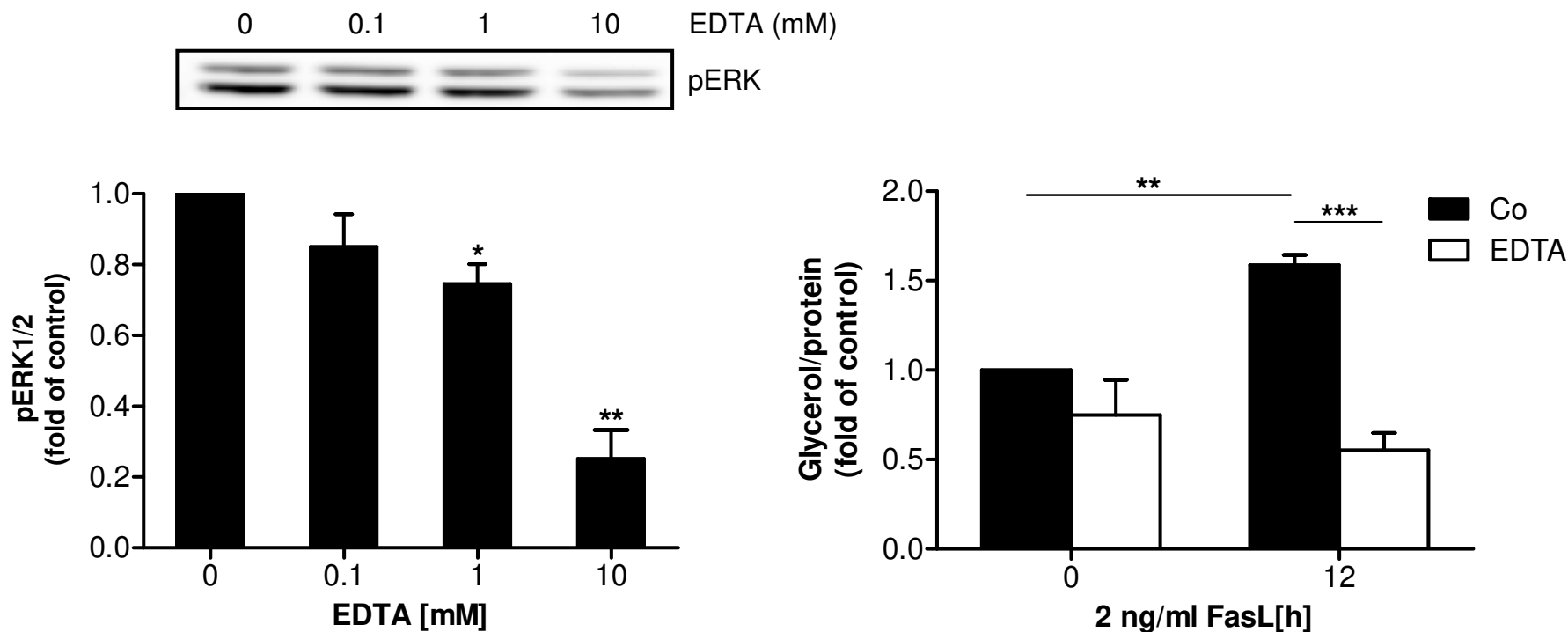
Supplemental Figure 6 Fas activation reduces perilipin protein levels ERK-independently

3T3-L1 adipocytes were treated with or without FasL (2ng/ml) in the presence or absence of the MEK inhibitor U0126 (50 μ M) as indicated. Lysates were resolved by LDS-PAGE and immunoblotted. Shown are representative blots and densitometry analyses of three independent experiments. Results are expressed relative to untreated controls. *Vertical white line* denotes deletion of unrelated bands from the same membrane for the clarity of the representation. * $p < 0.05$, ** $p < 0.01$.



Supplemental Figure 7 Fas activation does not activate ER stress in 3T3-L1 adipocytes

3T3-L1 adipocytes were treated with or without FasL (2ng/ml) or 10 μ g/ml Tunicamycin. Lysates were resolved by LDS-PAGE and immunoblotted for Grp78 and actin antibody, respectively. Results are the means \pm SEM of three independent experiments.



Supplemental Figure 8 EDTA blunts FasL-stimulated lipolysis

3T3-L1 adipocytes were incubated with 2ng/ml FasL and different concentrations of EDTA for 12 hours. **Left panel**, the amount of phosphorylated ERK1/2 was analyzed in whole cell lysates by Western blot technique. Shown are representative blots and quantification of four independent experiments. **Right panel**, glycerol release was determined in adipocytes treated with or without 2ng/ml FasL and 10 mM EDTA for 12 hours. Results are the means \pm SEM of four independent experiments.